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Transplantation of low dose $CD34^+Kdr^+$ cells promotes vascular and muscular regeneration in ischemic limbs

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ABSTRACT

Hematopoietic progenitor cell transplantation can contribute to revascularization of ischemic tissues. Yet, the optimal cell population to be transplanted has yet to be determined. We have compared the therapeutic potential of two subsets of human cord blood $CD34^+$ progenitors, either expressing the VEGF-A receptor 2 (KDR) or not. In serum-free starvation culture, $CD34^+KDR^+$ cells reportedly showed greater resistance to apoptosis and ability to release VEGF-A, as compared with $CD34^+KDR^-$ cells. When injected into the hind muscles in immunodeficient SCIDbg mice subjected to unilateral ischemia, a low number (10^3) of $CD34^+KDR^+$ cells improved limb salvage and hemodynamic recovery better than a larger dosage (10^4) of $CD34^+KDR^-$ cells. The neovascularization induced by KDR^+ cells was significantly superior to that promoted by KDR^- cells. Similarly, endothelial cell apoptosis and interstitial fibrosis were significantly attenuated by KDR^+ cells, which differentiated into mature human endothelial cells and also apparently skeletal muscle cells. This study demonstrates that a low number of $CD34^+KDR^+$ cells favors reparative neovascularization and possibly myogenesis in limb ischemia, suggesting the potential use of this cell population in regenerative medicine.

Key words: Endothelial progenitor cells • angiogenesis • vasculogenesis • ischemia • apoptosis • vascular endothelial growth factor.

Pioneer studies indicated that reparative angiogenesis is bolstered by recruitment of endothelial progenitor cells (EPCs) that functionally incorporate into sites of neovascularization and participate in ongoing collateralization (1). Depletion or functional abnormalities of these cells may contribute to cardiovascular disease progression and impaired

healing after ischemic injury (2–4). Transplantation of EPCs, isolated from adult peripheral blood (APB), cord blood (CB), or bone marrow (BM), has shown great promises as a potential tool to combat ischemic diseases in preclinical animal models (5–9). In addition, preliminary evidence suggests the potential clinical benefit of stem cell therapy for treatment of ischemic diseases in man (10–13). Yet, several important issues must be considered to improve the efficacy and diminish the potential side effects of this approach, including the identification of the optimal cell type for transplantation.

In preclinical and clinical trials, crude preparations of BM or PB mononuclear cells (MNCs) have been generically defined as EPCs, based on their capacity to incorporate acetylated LDL, adhere on fibronectin, and bind ulex-lectin (14). This definition has been questioned, thus leading to the introduction of more specific markers for identification of "bona fide" EPCs [e.g., CD34, AC133, vascular endothelial growth factor A receptor-2 (VEGFR2/Flk-1/KDR) and VE-cadherin] (14–16).

In the embryo, KDR is critical for differentiation of endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and hematopoietic cells (17). In human postnatal life, the small subset of $CD34^+KDR^+$ cells are enriched not only for EPCs (15), but also for hematopoietic stem cells (HSCs) and primitive hematopoietic progenitor cells (HPCs) (18), while depleted of more advanced HPCs (18). Furthermore, this subset comprises hemangioblasts endowed with self-renewal capacity and potential for differentiation into both hematopoietic and endothelial cells (19), as well as into other mesodermic tissues (unpublished results). Conversely, $CD34^+KDR^-$ cells essentially comprise HPCs.

So far, a side-by-side comparison of the in vivo therapeutic potential of different subpopulations of $CD34^+$ has been impaired by the low frequency levels of EPC- and HSC-enriched subfractions, e.g., only 1–2% $CD34^+$ cells stain for KDR. The present study, based on a large supply of $CD34^+$ cells from human CB, examined whether transplantation of low dose $CD34^+KDR^+$ cells into ischemic limbs may improve hemodynamic recovery and tissue healing better than treatment with $CD34^+KDR^-$ cells. In addition, we evaluated the contribution of transplanted KDR^+ cells in EC and skeletal myofiber regeneration, as well as in the protection from ischemia-induced apoptosis.

MATERIALS AND METHODS

Animals

All procedures complied with the standards stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD). Immunodeficient CB-17/IcrHsd-scid-bg mice (Harlan, Italy) were housed at constant room temperature (24±1°C) and humidity (60±3%).

Cell purification

CB was obtained from healthy, full-term placentas according to institutional guidelines. Low-density mononuclear cells (MNCs) (<1.077 g/ml) were isolated by Ficoll and $CD34^+$ cells purified by MACS column (Milteny, Bergisch Gladbeck, Germany). $CD34^+KDR^+$ and $CD34^+KDR^-$ subpopulations were sorted by FACSVantage-SE with specific anti-KDR MoAb

(19). The sorted cells were maintained in Iscove's medium containing 10% FCS at 4°C until used.

Induction of ischemia and EPC transplantation

With mice under the effect of 2,2,2-tribromoethanol anesthesia (880 mmol/kg body wt IP, Sigma, St. Louis, MO), the left femoral artery was exposed, dissected free, and excised (20). On the same occasion, human cord blood cells ($CD34^+KDR^+$ [10^3 or 2×10^3] or CD34+KDR^- [10^4], or vehicle (saline) were intramuscularly injected into ischemic limbs. The experimental setting is intended to mimic the clinical situation of patients with extensive, acute arterial occlusion.

Postischemic hemodynamic recovery

Hindlimb blood flow of anesthetized mice was measured by laser Doppler flowmetry (Perimed, Stockholm, Sweden) under basal conditions and then weekly after surgery, and the ratio of perfusion between ischemic to nonischemic foot was calculated (20).

Histological assessment of skeletal muscle microcirculation and fibrosis

At 21 days from ischemia, anesthetized mice were perfusion fixed, and limb muscles were processed for histological analyses. Interstitial fibrosis was morphometrically assessed in Azan Mallory-stained sections and expressed as percent of total muscle section. Vascular ECs were identified by immunostaining for factor VIII (Dako, Carpinteria, CA) or CD31 (JC/70A, CD31 mouse anti-human, Dako) and myofibers by immunostaining for WGA (Fluorescein-labeled Wheat Germ Agglutinin, Vector Laboratories, Burlingame, CA). For identification of arterioles, sections were stained with a mouse monoclonal anti-smooth muscle actin (Sigma). Arterioles were recognized as vascular structures containing one or more continuous layers of smooth muscle cells and morphometrically classified according to their luminal size as small (5 to 25 μm in diameter) or large arterioles (25 to 55 μm in diameter) (21). Immunostaining for proliferation-associated protein K_i-67 (K_i-67 mouse IgG1, Dako) was performed to identify the rate of EC regeneration.

Histological analysis was performed in a blinded fashion. Capillaries and myofibers were counted with an ocular reticle (9604 μm^2 area) at 1000× magnification. Twenty-five fields per section were randomly examined and averaged. The number of capillaries (n_{cap}) and myofibers (n_{fiber}) per field was used to compute capillary or myofiber numerical density per square millimeter of section. Because ischemia can produce myofiber shrinkage, changes in capillarity were also expressed by normalizing capillary density by myofiber density (n_{cap}/n_{fiber}). The number of arterioles (n_{art}) in the entire section was counted at 1000× magnification. Arteriole density per square millimeter of section (n_{art}/mm^2) was then calculated.

Apoptosis was assessed by TUNEL assay. TUNEL-positive cell number was counted in the whole area section at 1000× magnification and expressed as number of positive cells per square mm section or normalized by capillary or myofiber number.

Incorporation of CB-derived progenitor cells into skeletal myofibers

Immunohistochemical stainings were performed on 5 μm thick paraffin sections. Deparaffinized sections were incubated for permeabilization in PBS with 0.1% Triton X-100 for 3 min. After

rinse with PBS, tissue sections were exposed for 2 h at 37°C to mouse monoclonal anti-desmin or anti-dystrophin MoAb (DE-U-10, MANDY S8 respectively, Sigma) or rabbit polyclonal anti-laminin Ab (Sigma) in PBS, 3% bovine serum albumin (BSA) 0.05% Tween 20. After two washes in PBS, sections were incubated for 30 min at 37°C with antibodies against Rodamine Red (goat anti-mouse IgG (H^+L) or goat anti-rabbit IgG, Molecular Probes, Carlsbad, CA) in PBS 3% BSA 0.05% Tween 20. Sections were rinsed and incubated with fluorescein-conjugated anti-human nucleus Ag (HNAg) MoAb (MAB 1281, mouse IgG1, Chemicon International, Temecula, CA) diluted 1:50 in PBS 3% BSA 0.05% Tween 20 for 1 h. After two washes nuclei were counterstained using Hoechst 33342 (Molecular Probes). Slides were mounted using fluorescent mounting medium, and then observed and analyzed by confocal laser-scanning microscopy.

Statistical analysis

All results are expressed as mean ± standard error (SEM). Multivariate repeated-measures ANOVA was performed to test for interaction between time and grouping factor. In multiple comparisons in which ANOVA indicated significant differences, the statistical value was determined according to the Bonferroni's method. Differences within and between groups were determined using paired or unpaired Student's *t* test, respectively. The comparative incidence of limb salvage was evaluated by χ^2 analysis. A P value <0.05 was interpreted to denote statistical significance.

RESULTS

$CD34^+KDR^+$ cell transplantation enhances the neovascularization response to ischemia and protects ECs from apoptosis

In view of the unique resistance of $CD34^+KDR^+$ cells to starvation in vitro (22), as well as the marked enrichment of these cells for hematopoietic/endothelial primitive cells (15, 18, 19), we planned to transplant in mouse ischemic limb a low number of $CD34^+KDR^+$ cells (10^3 or 2×10^3/mouse) vs. a larger number of $CD34^+KDR^-$ (10^4/mouse). As shown in Fig. 1*A*, transplantation of 10^3 $CD34^+KDR^+$ cells increased *capillary density* of ischemic muscles by 114% (1408±96 vs. 659±28 cap/mm^2 in contralateral normoperfused adductors, P<0.01). No further increase in capillarity was observed by doubling the cell dosage. Comparatively, the angiogenic effect promoted by $CD34^+KDR^+$ cells largely exceeded (P<0.01) the increment seen in muscles injected with vehicle (66%), or 10^4 $CD34^+KDR^-$ (46%). Capillary to myofiber ratio averaged 1.25 ± 0.10 in ischemic muscles of $CD34^+KDR^+$ mice (corresponding to a 64% increment with respect to contralateral adductors, P<0.01), a figure significantly higher (P<0.05) than that observed in mice given vehicle (1.01±0.08, +33% vs. contralateral, P<0.05), or $CD34^+KDR^-$ subfraction (0.94±0.07, +24% vs. contralateral, P=N.S.). Thus, transplantation of a low number $CD34^+KDR^+$ cells enhanced spontaneous capillarization response to ischemia, while $CD34^+KDR^-$ cells were ineffective at 1-log higher number.

Figure 1*B* shows that arteriogenesis was potentiated by transplantation of $CD34^+KDR^+$ cells. Noteworthily, this effect was dosage-dependent; in fact, 10^3 $CD34^+KDR^+$ cells did not augment the spontaneous increase in arteriole density, while 2×10^3 potently stimulated arteriogenesis (33.6±6.5 art/mm^2, +352% vs. contralateral, P<0.01) as compared with vehicle (142%). In contrast, arteriole density of muscles injected with 10^4 $CD34^+KDR^-$ cells did not differ from

controls (P=N.S.). Morphometric analysis of neovascularization denoted a twofold increment in arteriole diameter in adductor muscles transplanted with $CD34^+KDR^+$ (35±7 μm) or $CD34^+KDR^-$ subfraction (29±6 μm) as compared with vehicle-injected (14±5 μm, $P<0.05$ for both comparisons).

Then, we investigated whether the increase in neovascularization can be attributed to differentiation of the injected primitive cells into mature vascular cells (Fig. 2*A*, *B*). Figure 2*A* illustrates positive controls (human skeletal muscle sections) stained with antibodies against markers of ECs or myofibers (upper panels) and counter-stained with antibodies against human nuclear antigen (HNAg) (lower panels). Figure 3*B* displays the results of immunohistochemical studies on muscular sections from transplanted mouse adductors. The results of cell counting performed on a total of 2000 capillaries (3 mice/group) revealed 9.0±1.2 $HNAg^+$ ECs in capillaries from $CD34^+KDR^+$ transplanted muscles, as compared with 2.1±0.2 for the $CD34^+KDR^-$ group ($P<0.01$). Abundant expression of Ki67, a proliferation-associated protein, was detected in differentiated ($HNAg^+$) or nondifferentiated ($HNAg^-$) ECs (Fig. 2*B*). These features were observed at 3 weeks from cell transplantation, suggesting that $CD34^+KDR^+$ derived ECs continue to proliferate at late stages of reparative process.

Absence of $HNAg^+$ cells in contralateral (negative controls, right panels of Fig. 2*B*) or vehicle-injected muscles confirms the specificity of the reaction.

To determine if transplanted CB cells incorporate into the arteriole wall, we screened skeletal muscle sections triple stained with HNAg Ab (green), smooth muscle actin Ab (red) and Hoechst nuclear dye (blue). Figure 2*C* illustrates immunohistochemical evidence of human nuclei incorporation into arterioles. Incorporation occurred mainly at the level of the endothelial layer (also identified by CD31 immunostaining, not shown), with rare $HNAg^+$ cells being detectable in arteriole muscular layer (stained in red by smooth muscle actin). The results of cell counting performed in a total of 200 arterioles (3 mice/group) revealed 1.0±0.1 human nuclear positive cells in vessels from $CD34^+KDR^+$ transplanted muscles, as compared with 0.6±0.1 in $CD34^+KDR^-$ ($P<0.05$). $HNAg^+$ cells were absent in vessels of muscles injected with vehicle. Similarly, no immunostaining for HNAg was detected in muscles contralateral to those injected with $CD34^+KDR^+$ or $CD34^+KDR^-$ (results not shown).

Finally, we evaluated whether CB cell therapy may combat ischemia-induced apoptosis (6 mice/group). TUNEL-positive ECs were significantly less in $CD34^+KDR^+$ injected adductors (3.8±0.9 cells/1000 cap), as compared with vehicle- or $CD34^+KDR^-$-injected ones (9.5±2.5 and 8.4±1.6 cells/1000 cap, $P<0.05$ for both comparisons) (results not shown).

$CD34^+KDR^+$ cell transplantation protects skeletal myofiber against apoptosis and causes muscle cell regeneration

As shown in Fig. 3, apoptosis was dramatically activated by ischemia at myofiber level in the vehicle group (69±1% of 3500 myofibers examined). This effect was reduced by $CD34^+KDR^+$ transplantation (8±1%, $P<0.01$) and to a significantly lesser extent by $CD34^+KDR^-$ cells (16±1%, $P<0.01$ vs. $CD34^+KDR^+$). Apoptosis was virtually absent in contralateral normoperfused muscles. Six mice were included in all groups.

We further analyzed the morphology of limb adductor muscles stained with Azan Mallory (4 mice/group). As shown in Fig. 4, interstitial fibrosis was increased in vehicle-injected ischemic muscles (54±3% of total section area) as compared with normoperfused contralateral ones (3±1%, $P<0.01$). Transplantation of $CD34^+KDR^+$ cells resulted in a marked attenuation of fibrosis (10±2%, $P<0.01$ vs. vehicle), whereas the anti-fibrotic effect of $CD34^+KDR^-$ cells, transplanted at a 1-log more elevated number, was significantly less pronounced (30±1%, $P<0.01$ vs. $CD34^+KDR^+$).

To determine the exact location of CB cell incorporation, we performed high-resolution, confocal analysis of skeletal muscle sections stained with antibodies raised against HNAg, dystrophin (a label for sarcolemma), laminin (labeling basal lamina), or desmin (a label for interstitial cells). Donor human nuclei were identified in muscles injected with CB cells underneath the sarcolemma (Fig. 5*A*), underneath the basal lamina (Fig. 5*B*), as well as in interstitial space (Fig. 5*C*) (4 mice/group). The results of cell counting performed in a total of 3500 skeletal myocytes indicate an increased incorporation of $CD34^+KDR^+$ cells vs. $CD34^+KDR^-$ cells into the myofibers (12.1±0.7 and 6.0±0.5 respectively, $P<0.01$) and underneath the basal lamina (8.0±0.9 and 4.0±0.7, $P<0.01$), while no group difference was observed with regard to incorporation into cells of the interstitial space (15.1±0.5 and 12.3±0.6, P=N.S.). In contrast, human nuclear positive cells were absent in vehicle-treated ischemic muscles. Similarly, no immunostaining for HNAg was detected in muscles contralateral to those injected with CB cells (results not shown).

$CD34^+KDR^+$ cell transplantation improves spontaneous hemodynamic recovery and clinical outcome

In immunodeficient mice, postischemic healing is severely compromised. Accordingly, 13 (75%) of 18 mice underwent spontaneous foot auto-amputation and the remainders showed an ischemic to contralateral blood flow ratio of 0.27±0.03 at 21 days. Clinical outcome remained unaffected by transplantation of 10^4 $CD34^+KDR^-$ cells: in fact, 7 (50%) of 14 underwent auto-amputation, with the remainders showing ischemic to contralateral ratio of 0.28±0.04 (P=N.S. vs. vehicle). In contrast, 2×10^3 $CD34^+KDR^+$ cell transplantation resulted in improved outcome as denoted by reduced auto-amputation rate (15% of 14 mice, $P<0.05$ vs. vehicle or $CD34^+KDR^-$ cells). In the remainders, hemodynamic recovery was ameliorated by $CD34^+KDR^+$ cell transplantation (as denoted by an ischemic to contralateral ratio of 0.40±0.05, $P<0.05$ vs. vehicle-treated or $CD34^+KDR^-$). Figure 6 illustrates representative laser Doppler images indicating improved hemodynamic recovery of mice transplanted with 2×10^3 $CD34^+KDR^+$ cells. A lower dosage (10^3 cells) produced a milder improvement of the clinical outcome (data not shown).

DISCUSSION

In the model of limb ischemia used here, transplantation of $1\text{-}2\text{x}10^3$ human CB $CD34^+KDR^+$ cells markedly stimulates spontaneous capillarization. Conversely, transplantation of a larger number (10^4) of $CD34^+KDR^-$ cells is largely ineffective. In the $CD34^+KDR^+$ cell group, $HNAg^+$ ECs detected in ischemic muscle at 3 weeks after transplantation showed a high proliferation capacity, as documented by coexpression of K_i-67: this finding newly demonstrates that cells derived from transplanted $CD34^+KDR^+$ EPCs continuously contribute to reparative angiogenesis up to late stages of the healing process.

Arteriogenesis is likewise potentiated by the proposed cell therapy approach (i.e., arteriole density was significantly increased by 2×10^3 $CD34^+KDR^+$ cells, whereas 10^4 $CD34^+KDR^-$ cells were ineffective). Furthermore, the HNAg assay showed that $CD34^+KDR^+$ cells incorporate into arterioles with higher frequency than $CD34^+KDR^-$ ones. Noteworthily, a cell dose-dependent effect was observed, in that 10^3 $CD34^+KDR^+$ cells exerted a negligible effect on arterial growth. Therefore, the dosage of 2×10^3 $CD34^+KDR^+$ seems to be the optimal one for therapeutic purposes.

It should be cautiously noted that the level of incorporation of human EPCs into resident endothelium is too low to account, by itself alone, for the improvement in reparative neovascularization. Additionally, a direct effect of the injected $CD34^+KDR^+$ cells on EC proliferation and viability via GF release might be considered. This trophic mechanism is supported by both in vitro and in vivo observations. In vitro studies indicated that $CD34^+KDR^+$ cells show a unique capacity to survive in serum-free starvation culture and to release relatively large amounts of VEGF-A, which reportedly exerts a trophic action on endothelial (23) and primitive hematopoietic cells (24). In vivo studies showed that $CD34^+KDR^+$ cell transplantation hinders apoptotic cell death and concomitantly reduces fibrosis in ischemic muscles. Recent evidence indicates that ischemia-mobilized EPCs, in the presence of risk factors, may differentiate in harmful cell phenotypes such as macrophages or fibrocytes (25). The possibility that transplanted $CD34^+KDR^+$ cells have contributed in fostering vasculogenesis and in preventing endogenous murine EPCs from assuming a proinflammatory, profibrotic phenotype was not assessed in this study, but deserves further investigation.

Furthermore, CB cell transplantation significantly stimulates muscle regeneration. This may derive from the improved reperfusion of the injured limb. Additionally, CB cells might have contributed directly to myogenic regeneration. A recent study documented that murine embryonic and human fetal ECs can differentiate into cardiac myocytes in vitro and in vivo (26). Specifically, EPCs from adult PB may differentiate into functionally active cardiomyocytes when cocultured with rat cardiomyocytes (27). The possibility that cell fusion rather than plasticity mediates these phenomena has been advocated (28). We observed that rare skeletal myocytes were positive for HNAg. Furthermore, expression of human MyoD gene was detected in murine muscles injected with $CD34^+KDR^+$ cells, thus suggesting trans-differentiation capacity of these cells into mature myocytes (unpublished results). The presence of $HNAg^+$ cells outside the fiber basal lamina suggests that $CD34^+$ cells could differentiate into myogenic cells in the interstitial space before being recruited into the satellite cell pool. Since the frequency of incorporation at the myofiber level was higher for the $CD34^+KDR^+$ subset, it is possible that cells derived from this subfraction have a greater capacity to organize within the regenerating muscle. However, the possibility that $HNAg^+$ positive myocytes could arise from cell fusion rather than from plastic differentiation should be considered.

More important, the present study documents that injection of $CD34^+KDR^+$ cells improves the clinical outcome of ischemic muscles and accelerates the rate of hemodynamic recovery. These results indicate that the microvasculature generated with the possible contribution of $CD34^+KDR^+$ cells was functionally efficient.

The number of EPCs required for therapeutic vasculogenesis is still unknown. It has been estimated that up to 12 L of autologous blood may be necessary to harvest EPCs in amounts sufficient to induce angiogenesis in patients after intravenous infusion (29). Risk factors,

including atherosclerosis and diabetes, can significantly alter EPC biology and thereby impose additional limitations to autologous transplantation. On the other hand, CB contains a larger number of EPCs than PB, while CB-derived EPCs have distinctive proliferative advantages, including a faster cell-cycle rate and longer telomeres (30). At present, human CB-derived EPC transplantation is negated by rejection triggered by host's immune defense response. In future studies autologous cryopreserved CB-derived EPCs could be used to combat ischemic complications, as already established for $CD34^+$ cells in the treatment of hematological disorders (31).

The therapeutic efficacy of a small number of $CD34^+KDR^+$ cells is a novel and important finding: It compares favorably with previous studies using markedly more elevated number of unselected progenitor cells (6, 29) (also transduced with VEGF in 29). The therapeutic efficacy of low dose $CD34^+KDR^+$ cells is seemingly mediated by diverse factors: 1) The $CD34^+KDR^+$ subfraction is enriched for EPCs/primitive hematopoietic cells and also comprises hemangioblasts (15, 18, 19); conversely, $CD34^+KDR^-$ cells largely comprise HPCs, while they are not enriched for EPCs/primitive hematopoietic cells and do not contain hemangioblasts (15, 19). It is not surprising, therefore, that the $CD34^+KDR^+$ cells transplanted in the ischemic area generate human ECs and possibly skeletal muscle cell, whereas the $CD34^+KDR^-$ subfraction has a markedly lower cell generation capacity. 2). $CD34^+KDR^+$ cells are exquisitely resistant to serum starvation (22); conversely, $CD34^+KDR^-$ cells rapidly die in these harsh culture conditions. Similarly, $CD34^+KDR^+$ (but not KDR^-) cells may be resistant to apoptosis when transplanted in the ischemic limb. 3) The release of relatively large amounts of VEGF-A by a small number of $CD34^+KDR^+$ cells might exert a significant anti-apoptotic and proliferative action on ECs in the transplanted area (22). Similar mechanisms may underlie the therapeutic effect of low dose $CD34^+KDR^+$ cell transplantation in a murine heart infarct model (23).

The $CD34^+KDR^+$ transplantation approach also triggers important questions unanswered so far. As previously mentioned, the $CD34^+KDR^+$ subset comprises not only EPCs (15), but also primitive hematopoietic cells (18) and hemangioblasts (19). The relative contribution of these functional cell subsets to the diverse ameliorative effects following $CD34^+KDR^+$ cell transplantation in the ischemic limb is not clear. It is noteworthy, however, that these functionally different cell populations may represent a single cell pool exhibiting hematopoietic and/or endothelial differentiation in different microenvironmental conditions (19). Also, the number of available $CD34^+KDR^+$ cells is limited. In this regard, ongoing studies in our laboratory aim to expand ex vivo the $CD34^+KDR^+$ subset, in order to facilitate its therapeutic use at the preclinical and then possibly at the clinical level.

Altogether, we propose that the $CD34^+KDR^+$ cell population may be considered as a potentially interesting tool for regenerative therapy in limb ischemia, particularly if coupled with a preliminary ex vivo expansion step inducing primitive cell self-renewal.

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Fig. 1

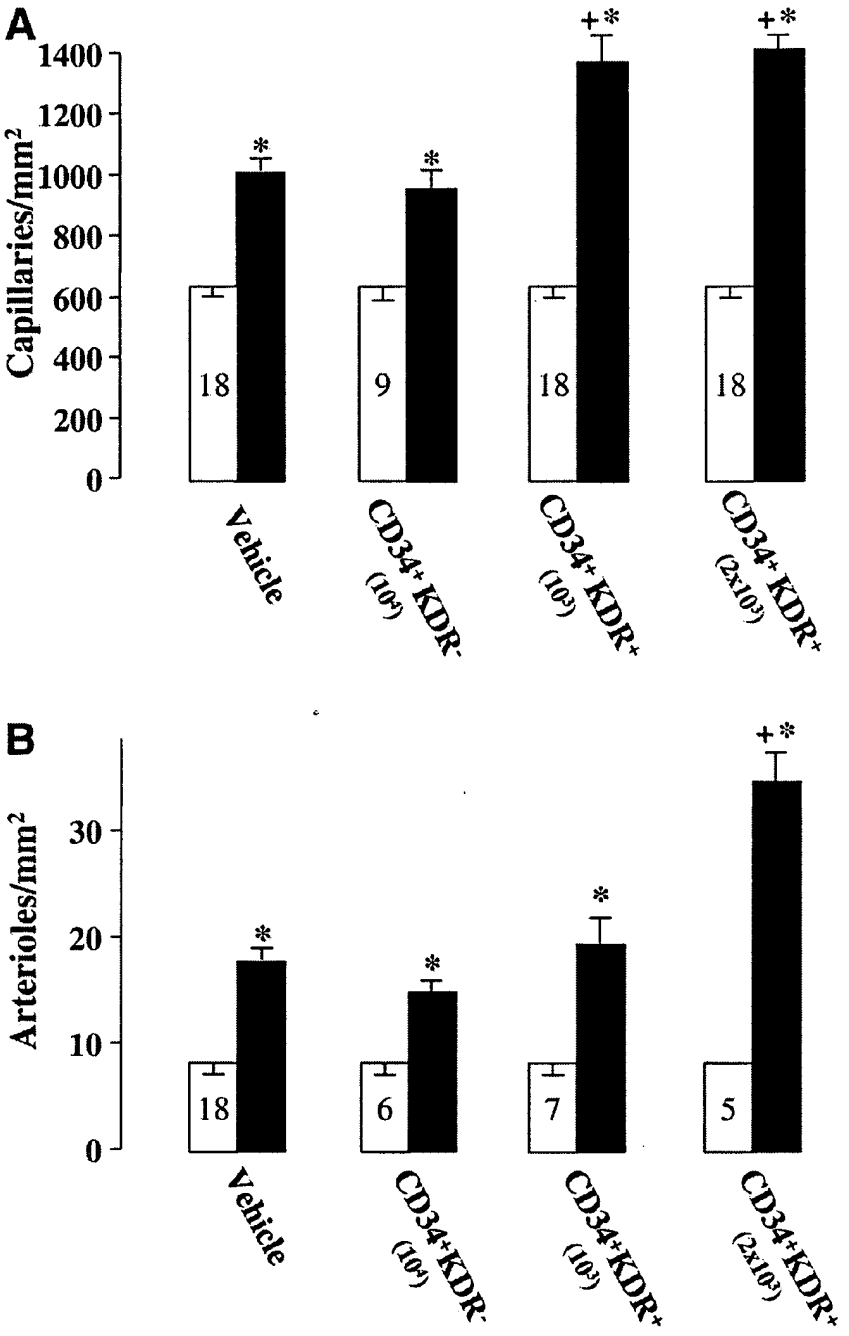

Figure 1. ***A***) Capillary density in ischemic adductor muscles and contralateral normoperfused ones (open columns). Mice underwent surgical excision of left femoral artery and had ischemic muscles injected with vehicle, cord blood-derived $CD34^+KDR^-$ (10^4) or $CD34^+KDR^+$ (10^3 or 2×10^3) cells. Capillary density of ischemic (full columns) and contralateral adductor skeletal muscles (open columns) was counted 21 days after induction of ischemia. Sample number is indicated within columns, and cell dosage is displayed on the abscissa. Values are mean ± SEM. $*P < 0.05$ vs. contralateral; $^+P < 0.05$ vs. vehicle and vs. $CD34^+KDR^-$. ***B***) Arteriole density in adductor muscles subjected to ischemia (full columns) and contralateral ones (open columns). Values are mean ± SEM. $*P < 0.05$ vs. contralateral; $^+P < 0.05$ vs. vehicle and vs. $CD34^+KDR^-$.

Fig. 2

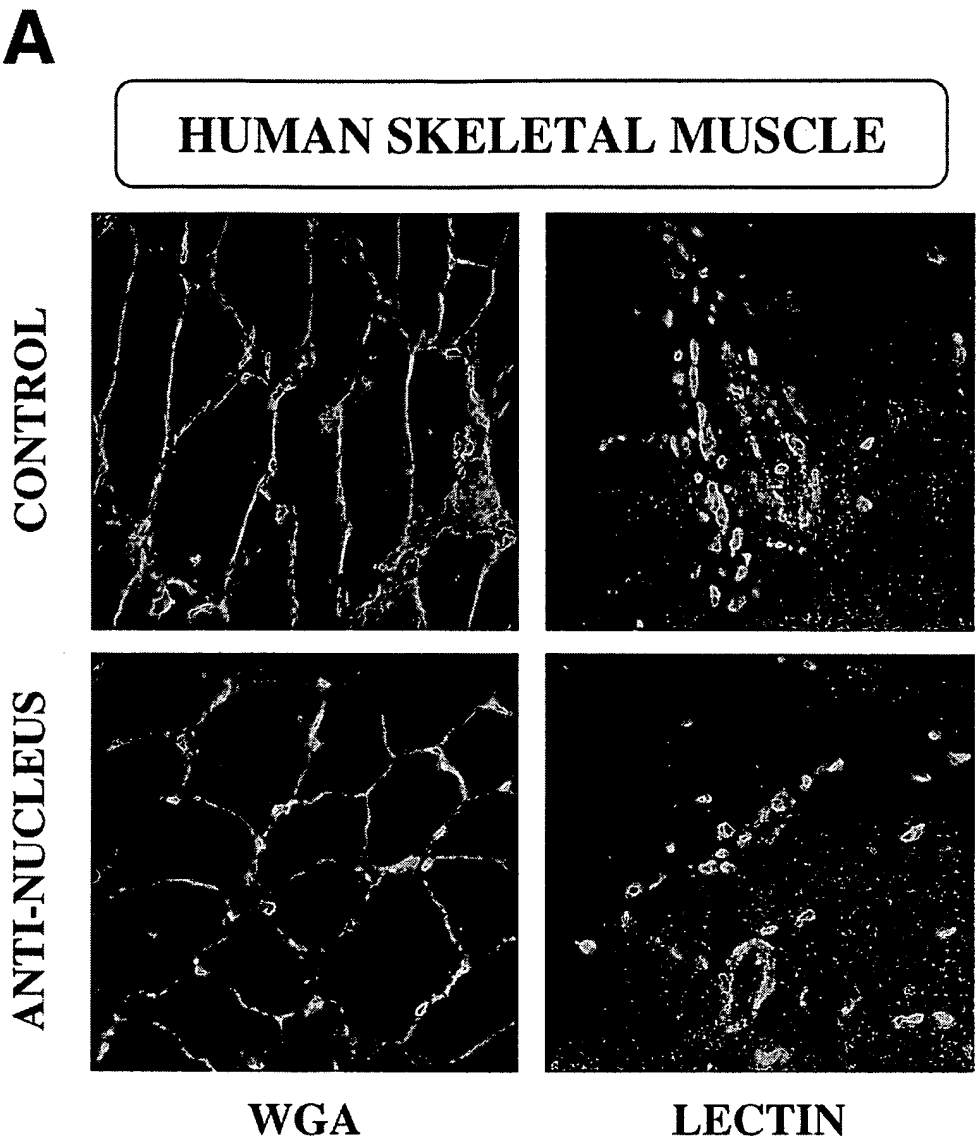

Fig. 2 (cont)

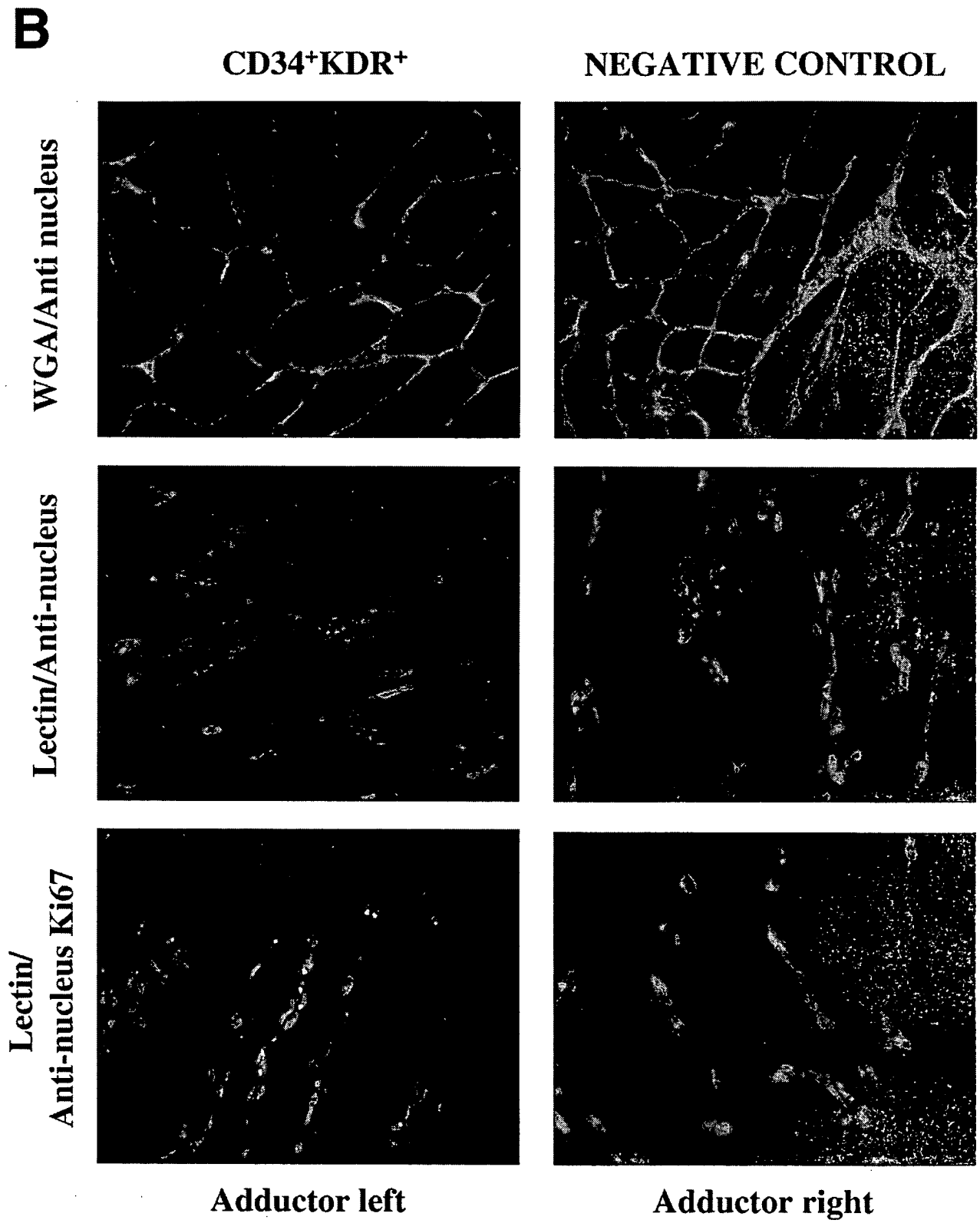
B
CD34+KDR+
NEGATIVE CONTROL
WGA/Anti nucleus
Lectin/Anti-nucleus
Lectin/
Anti-nucleus Ki67
Adductor left
Adductor right

Fig. 2 (cont)

C

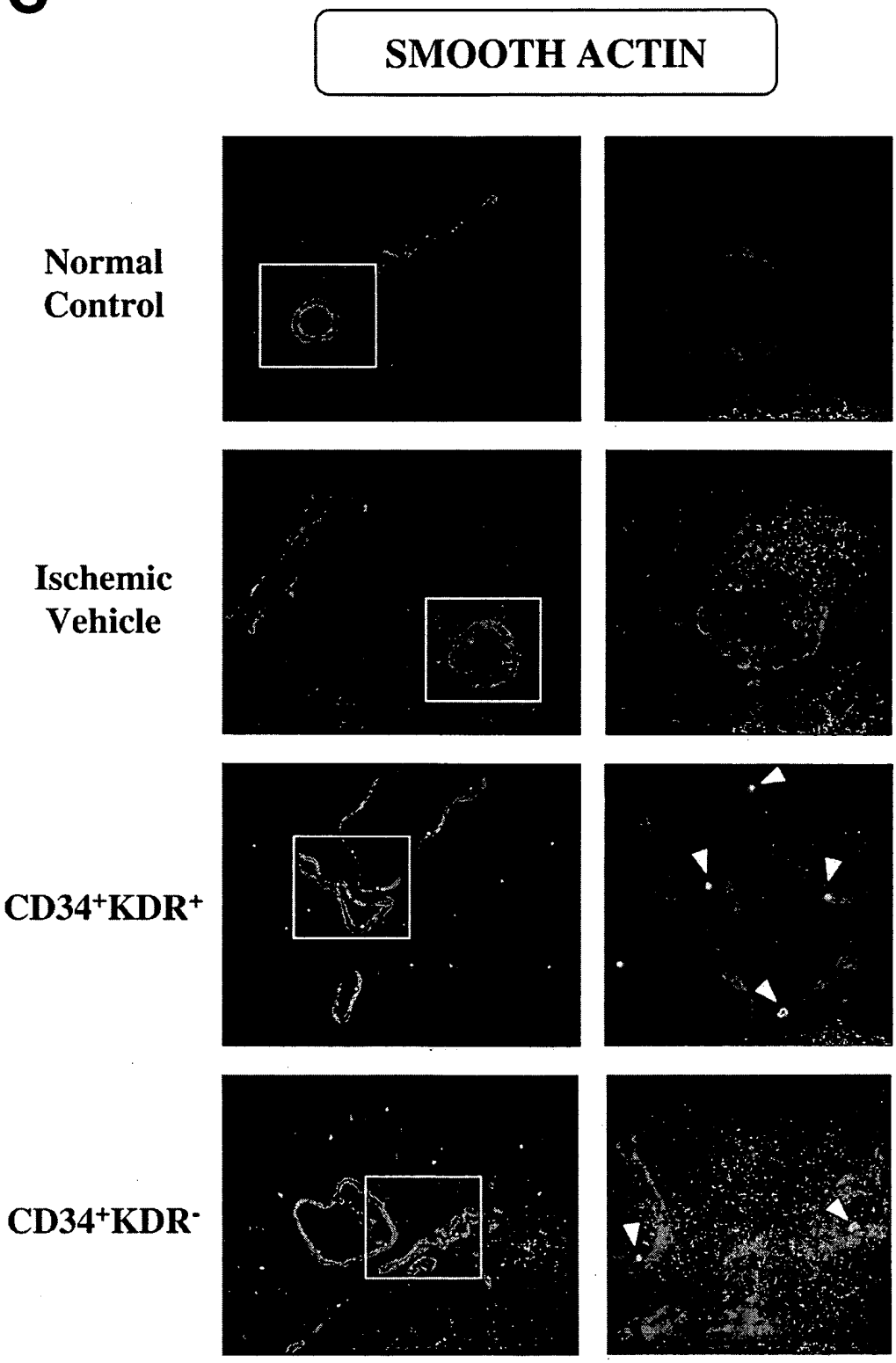
SMOOTH ACTIN
Normal Control
Ischemic Vehicle
CD34+KDR+
CD34+KDR-

Figure 2. ***A, B)*** Immunofluorescence analysis of expression of human nuclear antigen (HNAg) (red staining) on cryostat sections of human skeletal muscle counter-stained with antibodies raised against markers of skeletal myofibers (wheat germ agglutinin, WGA) or endothelial cells (lectin, also identified by CD31 immunostaining not shown) (***panel A***, positive control), as compared with mouse left ischemic adductor muscle implanted with $CD34^+KDR^+$ (***panel B***, Ad. Left). Contralateral normoperfused nonimplanted muscle is shown as negative control (***panel B***, Ad. Right). $HNAg^+$ ECs were found to incorporate into vascular tissue of ischemic mouse muscle. Proliferation-associated Ki67 (yellow staining) was detected in $HNAg^+$ or $HNAg^-$ ECs. Nuclear counterstaining, performed by Hoechst, is shown in all sections (blue staining). One representative of three independent experiments. ***C)*** Immunohistochemistry for smooth muscle actin (red) shows the presence of human nuclei stained in green incorporated into arterioles of mouse adductor muscle injected with $CD34^+KDR^+$ or $CD34^+KDR^-$. Several $HNAg^+$ ECs covering the arteriole and venule lumen (also identified by CD31 immunonostaining, not shown) are presented (arrowheads). Control normoperfused and vehicle-injected ischemic muscles were negative for human nuclei staining (not shown in figure). Nuclei counterstaining was performed by Hoechst (blue). One representative of 3 independent experiments.

Fig. 3

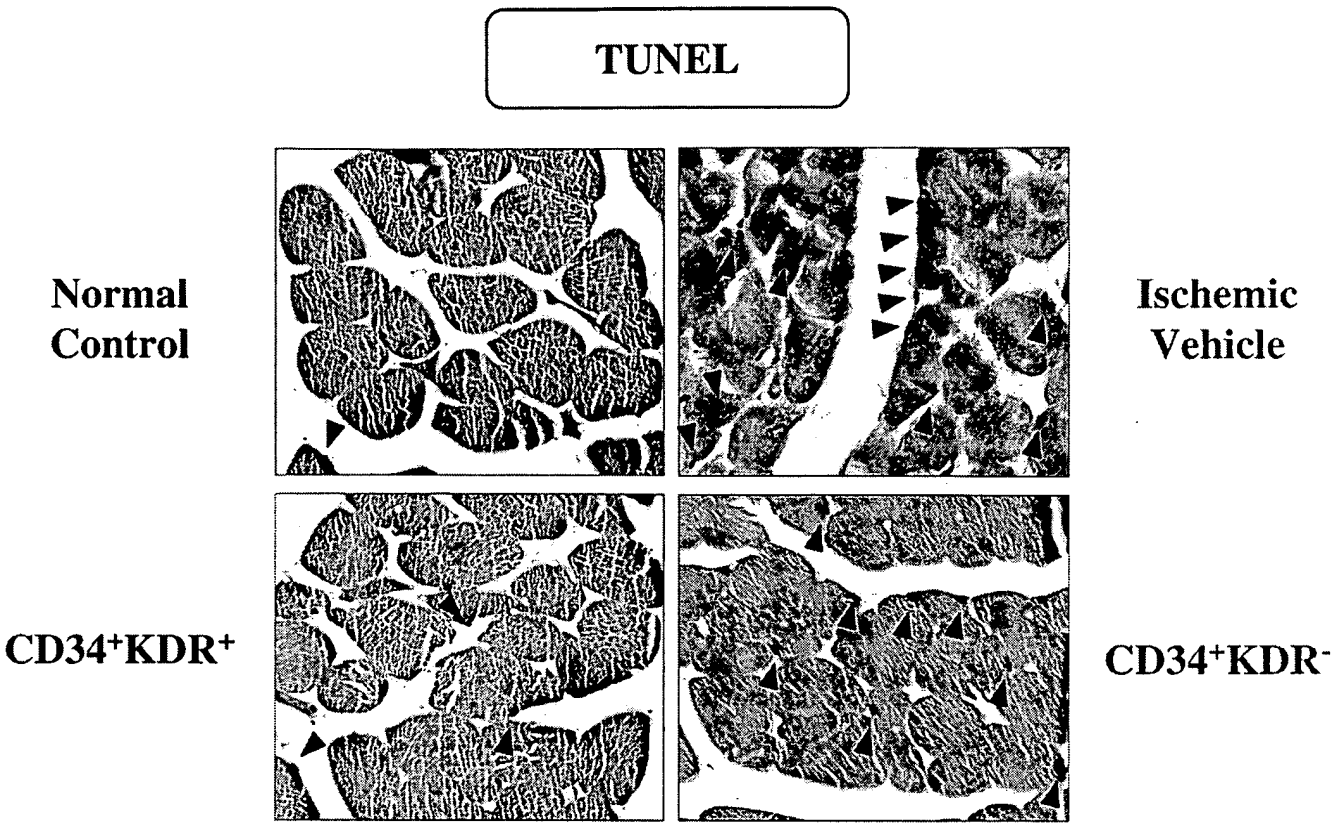

Figure 3. DNA breaks were detected by TdT-mediated dUTP nick end labeling (TUNEL) reaction (dark blue) and counterstained with eosin (red color). Black arrowheads indicate apoptotic muscle cells. Tunel staining shows the absence of apoptosis in muscle fibers in control normoperfused tissue, whereas increased levels of apoptosis are detected in ischemic vehicle tissue as a consequence of ischemia. A significant reduction of apoptotic events is observed in sections of mice injected with 2×10^3 $CD34^+KDR^+$ cells, while a less pronounced decrease is observed after transplantation of 10^4 $CD34^+KDR^-$ cells. One representative of 6 independent experiments.

Fig. 4

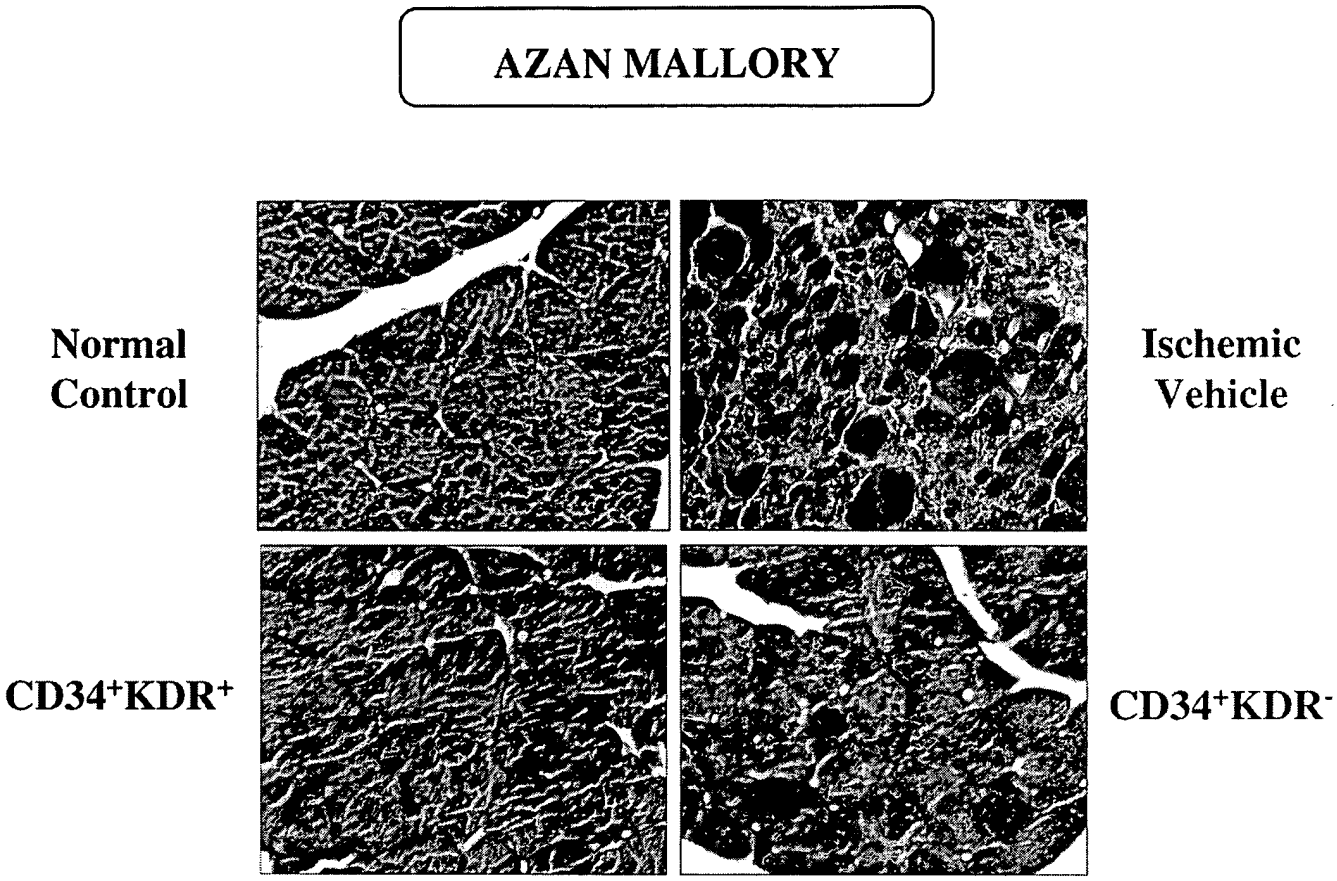

Figure 4. Azan Mallory staining shows the normal morphology of muscle fibers in control normoperfused tissue, whereas ischemic vehicle tissue displays relevant increase in fibrosis (blue). Interstitial fibrosis is significantly attenuated in skeletal muscles injected with 2×10^3 $CD34^+KDR^+$ cells. One representative of 4 independent experiments.

Fig. 5

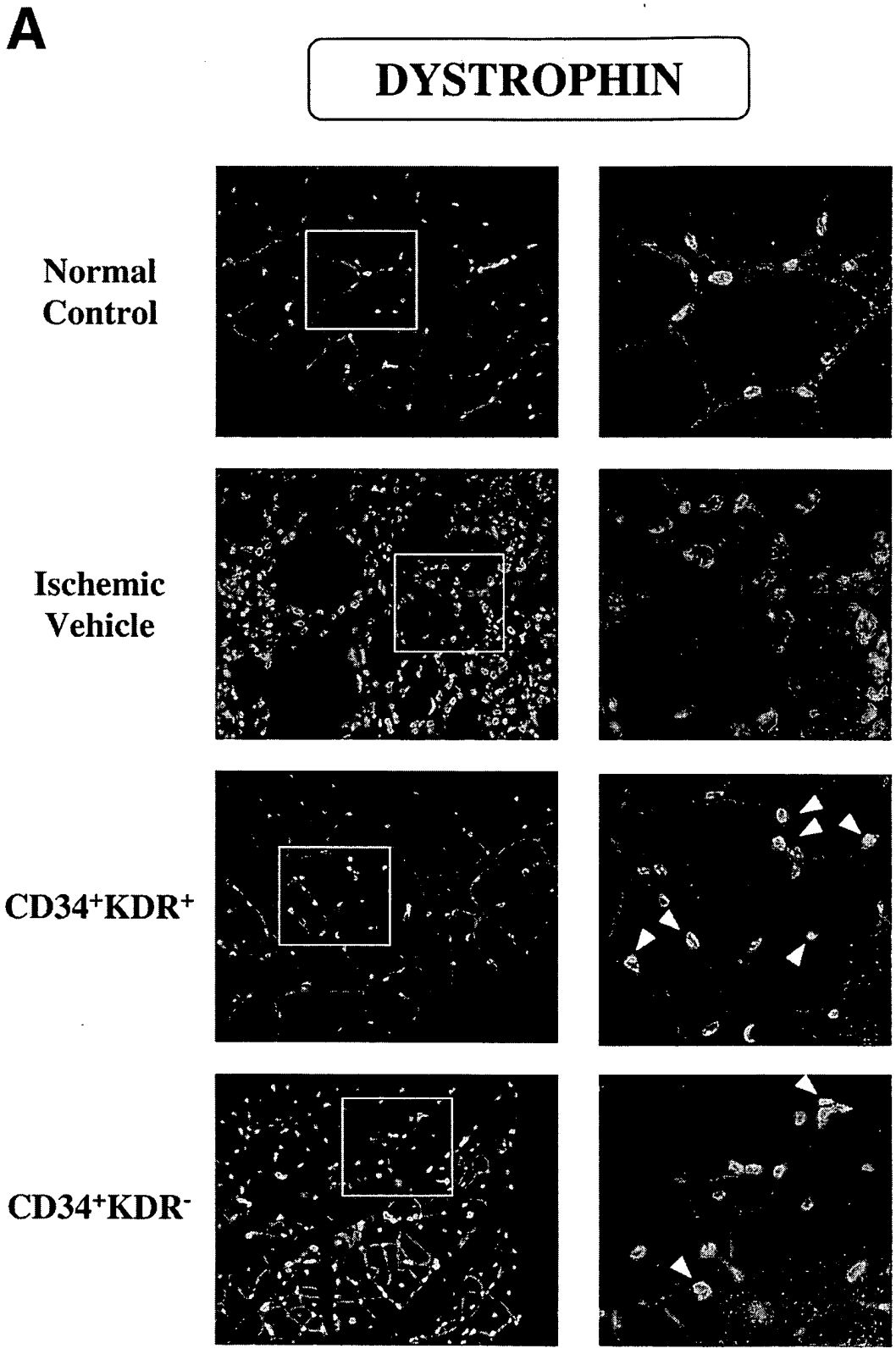
A
DYSTROPHIN
Normal Control
Ischemic Vehicle
CD34+KDR+
CD34+KDR-

Fig. 5 (cont)

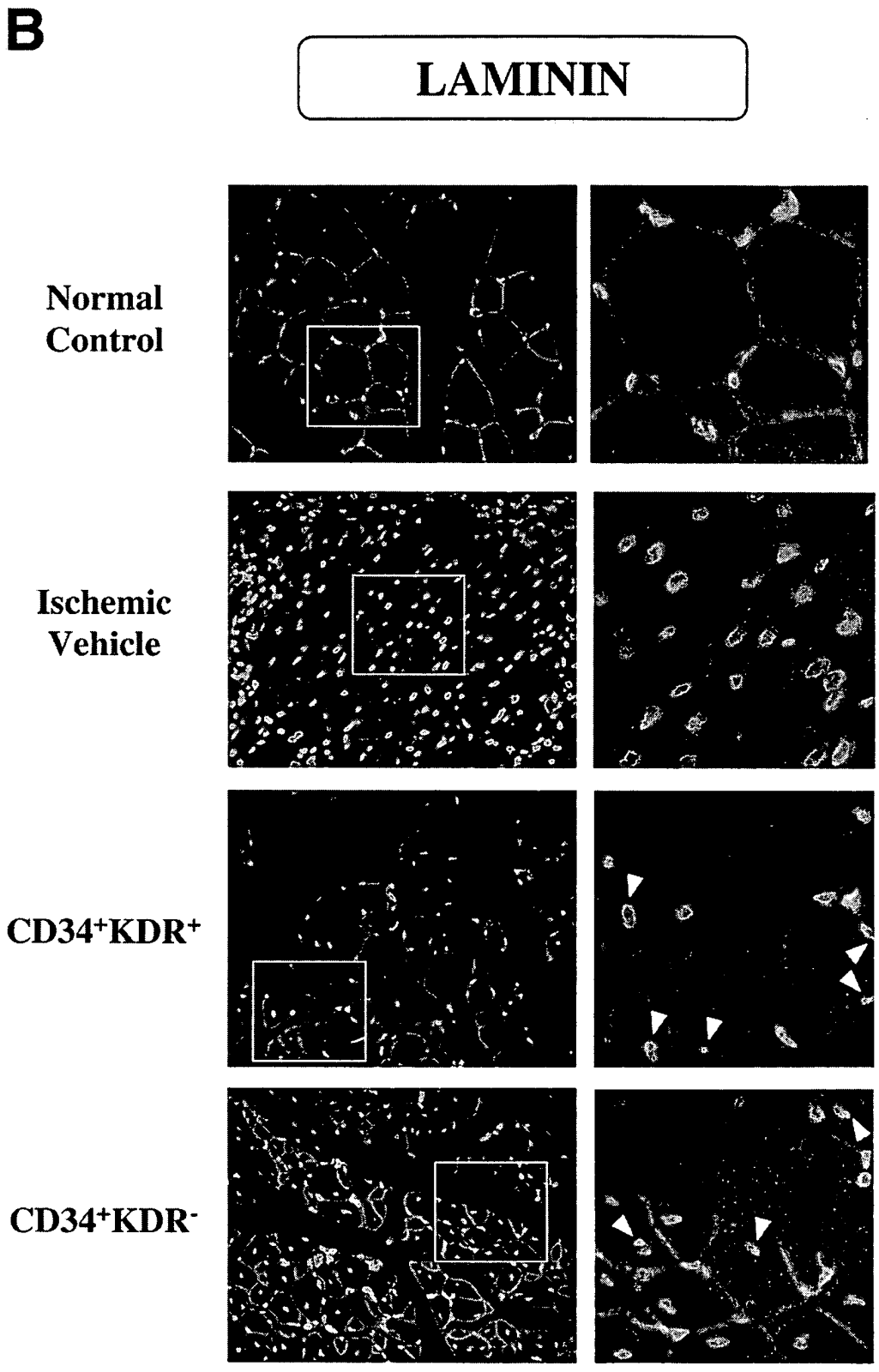
B
LAMININ
Normal Control
Ischemic Vehicle
CD34+KDR+
CD34+KDR-

Fig. 5 (cont)

C

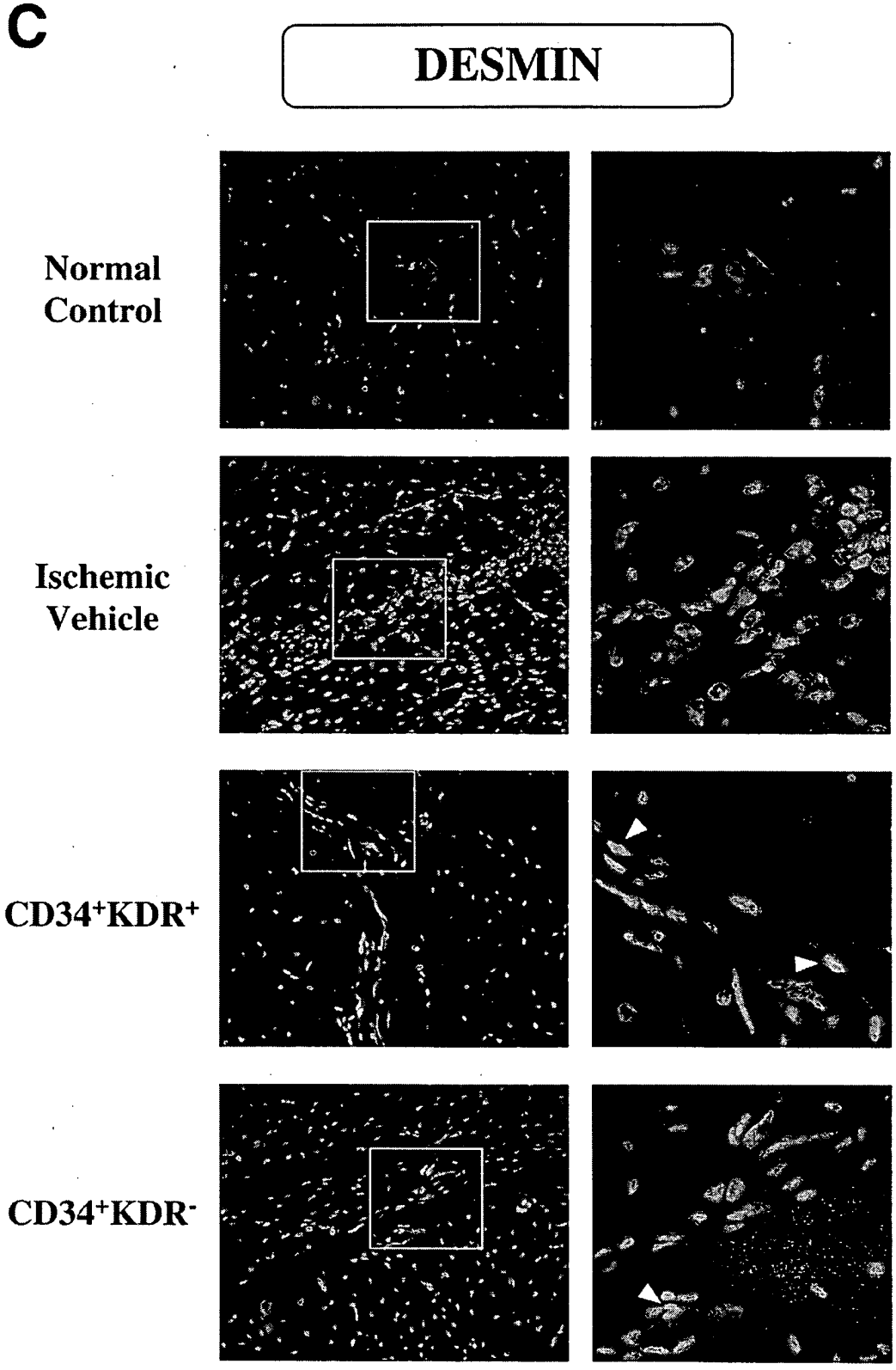

Figure 5. ***A)*** Immunofluorescence assay for dystrophin (that labels the sarcolemma, red) performed on paraffin section of mouse adductor muscles. Human $HNAg^+$ nuclei stained in green (arrowheads) are incorporated into the myofiber underneath the sarcolemma in mice whose ischemic adductor muscles were transplanted with $CD34^+KDR^+$ and $CD34^+KDR^-$ cells. One representative of four independent experiments. ***B)*** Immunofluorescence assay for laminin (a basal lamina marker, red) on paraffin section of mouse adductor muscles. $HNAg^+$ nuclei stained in green (arrowheads) are incorporated underneath the myofiber basal lamina. Nuclei counterstaining was performed by Hoechst (blue). One representative of four independent experiments. ***C)*** Immunohistochemical analysis of activated satellite cells, revealed by desmin staining, in paraffin section of adductor muscle (red). $HNAg^+$ nuclei stained in green (arrowheads) indicate human cells differentiated into satellite cells. Nuclei counterstaining was performed by Hoechst (blue). One representative of 4 independent experiments.

Fig. 6

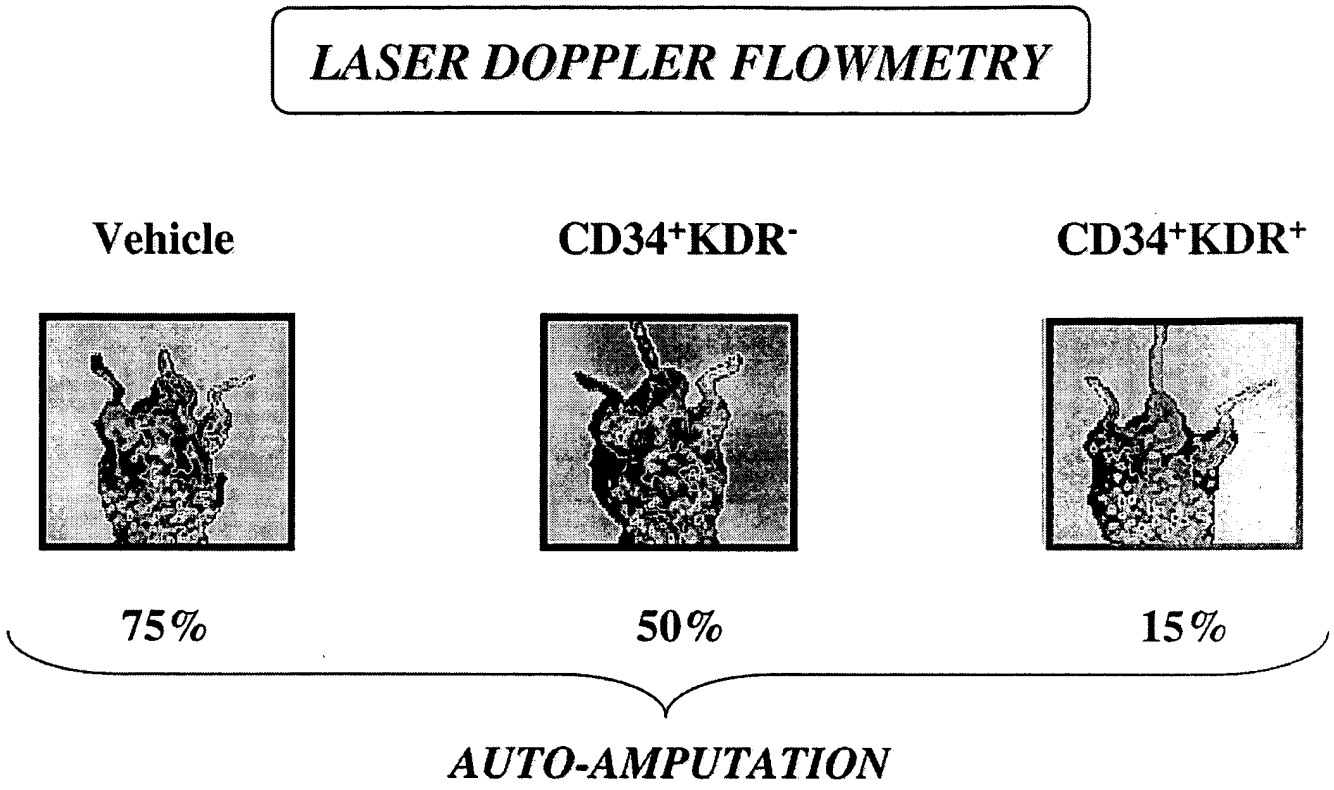

Figure 6. Representative laser Doppler images of improved hemodynamic recovery typical of $CD34^+KDR^+$ transplanted mice. Abdominal area and ventral parts of limbs and tail are shown. Colors correspond to 6 intervals of perfusion from 0% (dark blue) to 100% (red). Each group consisted of at least 14 mice.